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Persistence of Hepatitis C Virus RNA in Established Human Hepatocellular Carcinoma Cell Lines

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The persistence of the viral RNA of hepatitis C virus (HCV) was examined in 13 hepatocellular carcinoma (HCC) and two hepatoblastoma cell lines by reverse transcription followed by the polymerase chain reaction (RT-PCR). HCV RNA was detected in three HCC lines (JHH-1, JHH-4, and JHH-6) and negative-strand viral RNA was found in JHH-4, indicating that there is a putative replicative intermediate of HCV in JHH-4 cells. To rule out the possibility of contamination, the partial nucleotide sequences of HCV-specific PCR products of these three cell lines were determined. The clone from JHH-1 belonged to genotype 1 (1a or 1b), and the clones from JHH-4 and JHH-6 belonged to genotype 2b, but their sequences differed from each other. These cell lines may be useful for studies related to HCV.

KEY WORDS: cultured human hepatoma, HCV, PCR

INTRODUCTION

Hepatitis C virus (HCV) is a positive-strand RNA virus [Choo et al., 1989] and has been classified into its own genus within the family *flaviviridae* [Wengler, 1991]. Like other members of this family, HCV is thought to replicate through the production of a complementary negative-strand RNA as a template [Chambers et al., 1990; Cleaves et al., 1981]. Indeed, both positive and negative HCV RNA strands have been found in the livers of patients with HCV infection [Fong et al., 1991; Gunji et al., 1994; Sakamoto et al., 1994; Takehara et al., 1992]. Since the genome of HCV shows a high degree of heterogeneity [Bukh et al., 1994], several methods and different terms have been used to classify HCV genotypes [Cha et al., 1992; Chan et al., 1992; Nakao et al., 1991; Okamoto et al., 1992b]. Simmonds et al. [1994] recently proposed a detailed classification of HCV, which was used in the communication.

In spite of recent advances in the study of HCV in the molecular and biological field, a vaccine for this virus has not yet been developed. This is primarily due to hypervariability of the virus [Enomoto et al., 1994a,b; Martell et al., 1992]. Another problem is that, except for humans, only the chimpanzee can be infected with HCV. For these reasons, it is difficult to examine the effects of various antiviral substances such as interferon (IFN) and antihypervariable region 1 (HVR1) antibody (a putative neutralizing antibody) on HCV [Choo et al., 1994; Enomoto et al., 1994a,b; Farci et al., 1994; Kato et al., 1993, Kojima et al., 1994; Kurosaki et al., 1993; Sekiya et al., 1994; Taniguchi et al., 1993]. Moreover, many questions about HCV remain, including the mechanism of replication, the relationship between genotype and chronic infection, and the effects of mutation on development of hepatitis into liver cirrhosis (LC) or hepatocellular carcinoma (HCC).

It is therefore important to establish an in vitro system for propagating HCV. Lanford et al. [1994] developed an in vitro system for infection and replication of HCV using primary chimpanzee hepatocyte cultures. However, permanent human liver cell lines, which have a potential for infection and replication of HCV, also may be useful. We detected persistence of the 5′ untranslated region (UTR) of HCV RNA in three human HCC cell lines (JHH-1, JHH-4, and JHH-6) by screening 13 HCC cell lines and two hepatoblastoma (HB) cell lines.

MATERIALS AND METHODS

Cell Lines

To detect the presence of HCV, 13 HCC cell lines (HuH-7, HLE, PLC/PRF/5, Hep 3B, huH-1, huH-2,

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huH-4, JHH-1, JHH-2, JHH-4, JHH-5, JHH-6, and JHH-7) and two hepatoblastoma (HB) cell lines (HuH-6 Clone 5, and Hep G2) were screened. The cell lines JHH-1, JHH-2, JHH-4, JHH-5, JHH-6, and JHH-7 were sent to our laboratory by Dr. Seishi Nagamori (First Department of Internal Medicine, Jikei University School of Medicine where these cell lines were originally established) for the purpose of depositing them in the Japanese Cancer Research Resources Bank (JCRB). After examination of cellular characteristics, these cell lines were deposited in JCRB in 1994 and are now available to the scientific community. The characteristics and references of these cell lines are shown in Table I. Some of these HCC cell lines were derived from patients with HBV infection and have HBV DNA integrated in their genomes, but it was uncertain whether the tested cell lines came from patients with HCV infection. Cells were cultured in Dulbecco's modified Minimum Essential Medium supplemented with 10% fetal bovine serum.

Serum Sample

HCV RNA positive serum was obtained from a patient with HCV infection and used as a positive control. To avoid contamination, only one serum sample was used in this study. The amplified DNA from this serum was also used as a template for the HCV probe.

Oligonucleotide Primers

PCR primers and sequencing primers were synthesized based on the previous report of Okamoto et al. [1990a] who used the 5′UTR, known to be highly conserved among different HCV genotypes [Okamoto et al., 1990a; Simmonds et al., 1993]. In the first stage of PCR, we used an external primer pair, #32 (nt 45-64: 5′-CTGTGAGGAACTACTGTCTT-3′) as a sense primer and #36 (nt 246-265: 5′-AACACTACTCG-GCTAGCAGT-3′) as an antisense primer. In the second stage of PCR, an internal primer pair, #33 (nt 63-82: 5′-TTCACGCAGAAAGCGTCTAG-3′) was used as a sense primer and #48 (nt 188-207: 5′-GTTGATCCAA-GAAAGGACCC-3′) as an antisense primer. The same primers were used for sequencing.

RNA Extraction From Cell Lines and Serum

RNAs from cell lines and serum were extracted by the acid guanidinium thiocyanate-phenol-chloroform method [Chomczynski and Sacchi, 1987]. Briefly, semiconfluent growing cells were scraped from 10-cm dishes into 1.5 ml of the guanidinium buffer [4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sodium *N*-lauryl sarcosinate, 0.1 M 2-mercaptoethanol] and RNA was extracted with water-saturated phenol and chloroform. The extracted RNA was resolved in 300 μl of double-distilled water (ddw) treated with diethyl pyrocarbonate (DEPC-treated ddw). RNA extraction from serum was done almost in the same way, except that 200 μl of serum was mixed with 400 μl of the 1.5 times concentrated guanidinium buffer and that RNA was precipitated by the addition of ethanol with 12 μg of glycogen as a carrier and resolved in 6 μl of DEPC-treated ddw.

Reverse Transcription of HCV RNA

Five micrograms of RNA samples extracted from each cell line or 3 μl of RNA solution extracted from serum was denatured with 10 pmol of external antisense primer (#36) at 70°C for 10 min, annealed at 53°C for 1 min, and quickly chilled in ice water. These samples were made up to 10 μl, thereby including 100 units of SuperScript II (Gibco BRL, Grand Island, NY), 10 units of RNase inhibitor (Toyobo, Osaka), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol (DTT), and 250 μM of each deoxyribonucleoside triphosphate (dNTPs). The mixture was incubated at 37°C for 60 min.

Amplification of cDNA by Nested PCR

Ten microliters of the post-RT solution was made up to 20 μl, thereby including 0.5 units of *Taq* DNA polymerase (AmpliTaq, Perkin-Elmer Cetus Norwalk, CT), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 250 μM of each dNTP, and 500 nM external sense primer (#32). For screening cell lines, the first stage of PCR consisted of 40 cycles of denaturation at 94°C for 1 min, annealing at 45°C for 1 min, and extension at 72°C for 1 min. Then 1/10 volume of the first PCR products was reamplified using 500 nM of each internal primer pair (#33 and #48) for another 30 cycles under the same reaction conditions as the first, except for primer annealing at 55°C. The amplification of HCV RNA from serum as a positive control was done almost in the same way, except that the first stage of PCR was performed for 30 cycles, and the second was done for 20 cycles. The procedures recommended by Kwok and Higuchi [1989] were applied strictly to avoid contamination.

Ten microliters of the amplified products from each cell line and 1 μl of that from HCV RNA positive serum were size-fractionated in 3% agarose gel by electrophoresis and visualized with ethidium bromide staining under UV light. The expected size of these amplified DNA fragments was 145 bp.

Southern Blot Hybridization of Amplified Products

The second PCR products electrophoresed in 3% agarose gel were transferred to a nylon membrane (Hybond-N+, Amersham, Buckinghamshire, UK). The template DNA for the HCV probe was prepared by purification of the second PCR products amplified from the HCV positive serum, and labelled with [α-^{32}P]dCTP using a random primer kit (High Prime, Boehringer-Mannheim, Mannheim, Germany).

Direct Sequencing of PCR Products

Portions expected to contain the second PCR products were excised from the electrophoresis gel. The second PCR products were eluted from the pieces of the gel with Micropure (Amicon, Lexington, MA) and then fur-

TABLE I. Characteristics of 13 HCC and 2 HB Cell Lines

	Origin[a]	Age/Sex of patient[b]	Serum HBs-Ag[c]	Integration of HBV[d]	Production of HBs-Ag	References
HuH-7	HCC	57 yr/M	–	–	–	Nakabayashi H, et al. *Cancer Res* 1982;42:3858–3863.
HLE	HCC	68 yr/M	N.D.	–	–	Doi I, et al., *Jpn J Cancer Res* (Gann) 1975;66:385–392.
PLC/PRF/5	HCC	24 yr/M	+	+	+	Alexander JJ, et al. *S Afr Med J* 1976;50:2124–2128.
Hep 3B	HCC	8 yr/M	N.D.	+	+	Aden DP, et al. *Nature* 1979;282:615–616.
huH-1	HCC	53 yr/M	+	+	+	Huh N, et al. *Jpn J Cancer Res* (Gann) 1981;72:178–179.
huH-2	HCC	N.D.	N.D.	+	N.D.	Huh N, et al., unpub.
huH-4	HCC	51 yr/M	+	+	+	Huh N, et al. *Jpn J Cancer Res* (Gann) 1981;72;178–179.
JHH-1	HCC	50 yr/M	–	–	–	Homma S. *Jikeikai Med J* 1985;32:289–315.
JHH-2	HCC	57 yr/M	–	–	–	Homma S. *Jikeikai Med J* 1985;32:289–315.
JHH-4	HCC	51 yr/M	–	–	–	Homma S. *Jikeikai Med J* 1985;32:289–315.
JHH-5	HCC	50 yr/M	–	–	–	Nagamori S, et al. *HUMAN CELL* 1988;1(4):382–390.
JHH-6	HCC	57 yr/F	–	–	–	Nagamori S, et al. *HUMAN CELL* 1988;1(4):382–390.
JHH-7	HCC	53 yr/M	+	+	–	Homma S, et al. *HUMAN CELL* 1990;3(2):152–157.
HuH-6 Clone 5	HB	1 yr/M	–	–	–	Doi I. *Jpn J Cancer Res* (Gann) 1976;67:1–10.
Hep G2	HB	15 yr/M	N.D.	–	–	Aden DP, et al. *Nature* 1979;282:615–616.

[a]Whether these cell lines were derived from patients with HCV infection is unclear at present, because there were no methods to determine HCV infection at the time the cultures were initiated. HCC, hepatocellular carcinoma; HB, hepatoblastoma.
[b]M, male; F, female.
[c]Hepatitis B surface antigen.
[d]Hepatitis B virus.
N.D. = not described.

ther amplified to a level sufficient for sequencing. To avoid misreading by *Taq* polymerase, three separately amplified products were prepared for each cell line and directly determined their nucleotide sequences by the dideoxy-nucleotide chain-termination method (Amplitaq Cycle Sequencing Kit, Perkin-Elmer Cetus) [Innis et al., 1988]. The homology of the three amplified products was then verified.

Southern Blot Hybridization and Amplification by Nested PCR of Cellular DNA

Integration of the HCV genome into the host genome was examined by Southern analysis. Briefly, the extracted cellular DNAs from cell lines were digested with *Hin*dIII, which does not cleave the 5′UTR of HCV RNA. The DNA fragments were separated by electrophoresis in 0.8% agarose gel and transferred to a nylon membrane for hybridization with the ^{32}P-labelled probe. Integration of the HCV genome into the host genome was examined further by nested PCR. The extracted cellular DNA (0.2 μg) was amplified under the same reaction conditions as amplification of HCV cDNA from each cell line. The PCR products were visualized in the agarose gel after staining with ethidium bromide. For confirmation, they were transferred to a nylon membrane, and then analyzed by Southern blot hybridization.

Strand-Specific RT-PCR

To confirm whether HCV could replicate in the cell lines, reverse transcription was carried out under the same reaction conditions as described above, except for using 10 pmol of external antisense primer (#36) to detect positive-strand HCV RNA or external sense primer (#32) to detect negative-strand HCV RNA. After reverse transcription, the reaction mixture was heated at 94°C for 3 min, quickly chilled in ice water, and then incubated with 100 μg/ml of RNase A at 37°C for 90 min to degrade residual RNA template.

Nested PCR was also carried out under the same conditions as described above, except for using 500 nM external sense primer (#32) to detect positive-strand HCV RNA or external antisense primer (#36) to detect negative-strand HCV RNA at the first stage of PCR. Specificity to detect the negative strand was verified previously using recombinant positive-strand HCV RNA.

RESULTS

Detection of HCV RNA in Cell Lines by RT-PCR

The second PCR products were size-fractionated by electrophoresis in 3% agarose gel and hybridized with the ^{32}P-labelled probe. As shown in Figure 1, the 5′UTR of the HCV genome was detected in three HCC cell lines (JHH-1, JHH-4, and JHH-6). Attempts were also made to detect the core region of HCV. This region is known to be conserved fairly well, but it is a little more variable than the 5′UTR of the HCV genome. Although it was found that some of the amplified products were near the expected size of the HCV genome,

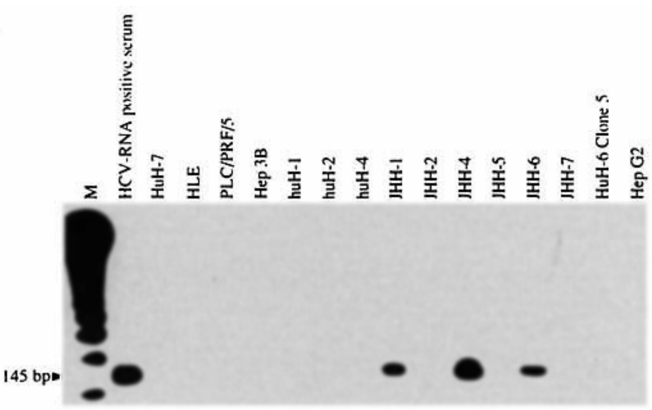

Fig. 1. Screening of 13 HCC cell lines and two HB cell lines for intracellular HCV RNA. RNAs extracted from each cell line were amplified by RT-PCR. The second PCR products were size-fractionated by electrophoresis in 3% agarose gel and transferred to a nylon membrane, and then they were hybridized with the ^{32}P-labelled probe. Lane M represents a 100-base DNA ladder (Pharmacia, Uppsala, Sweden). Arrowhead indicates the position of 145 bp DNA fragments, which is the expected size of amplified HCV RNA.

many nonspecific bands also appeared (data not shown). This region was therefore not examined in detail.

Sequencing of HCV-Specific RT-PCR Products From Cell Lines

To rule out contamination between the samples, especially with HCV RNA positive serum, the nucleotide sequences of the PCR products were determined. To avoid misreading by *Taq* polymerase, the homology of three separately amplified products from each cell line was verified. In Figure 2, the results are shown in contrast to the representative four HCV clones [Choo et al., 1991; Okamoto et al., 1991, 1992b; Takamizawa et al., 1991]. The sequences of the clones from JHH-1 and HCV RNA positive serum were the same and they belonged to genotype 1 (1a or 1b). However, the clone from JHH-4 obviously belonged to genotype 2b, showing its typical sequence. The clone from JHH-6 also belonged to genotype 2b. However, the sequence differed slightly from that of the JHH-4-derived clone. From these findings, it is obvious that JHH-6 has a different HCV clone from JHH-4.

Integration of HCV Genome Into Host Genome

Integration of the HCV genome into the host genome was examined in these three HCC cell lines by Southern analysis and amplification by nested PCR. Both results showed that there was no such integration (data not shown).

Detection of Negative-Strand HCV RNA

The possibility of replication of HCV in these three HCC cell lines was examined by strand-specific RT-PCR. As shown in Figure 3, negative-strand HCV RNA was detected in JHH-4, but not in JHH-1 or JHH-6. The detection of negative-strand HCV RNA in JHH-4 was

not always positive. Interestingly, even when positive-strand HCV RNA could not be detected the negative-strand was found.

Morphology and Characteristics of Cells

As shown in Figure 4, the cells are polygonal in shape and resemble hepatocytes. JHH-1 secretes carcinoembryonic antigen, JHH-4 secretes α-fetoprotein and albumin, and JHH-6 secretes ferritin in culture medium. The correlation between the differentiated state of the cell lines examined and the detection of HCV RNA was not clear.

DISCUSSION

There were no reports of HCC cell lines infected with HCV. In the present study, the 5′UTR of HCV RNA was detected in three HCC cell lines: JHH-1, JHH-4, and JHH-6. Although the human T-cell lines MOLT-4 Ma and HPB-Ma supported the replication of HCV for ~2 months, no persistent in vitro infection of HCV was observed [Shimizu et al., 1992, 1993, 1994; Shimizu and Yoshikura, 1994]. Yoshizawa et al. [1992] established a new HCC cell line, SUHC-1 from an HCV-positive patient, but no complete HCV could be detected in the cell line.

Many HCC without HBV infection are associated with HCV [Ohkoshi et al., 1990; Saito et al., 1990]. Thus some HCC cell lines established from patients without HBV infection have possibly been derived from those with HCV infection. However, the HCV genome cannot be integrated into the host genome because it is an RNA virus, although a remote possibility of illegitimate reverse transcription of HCV RNA and subsequence migration of cDNA remains. The results of Southern analysis showed no integration. However, whether the HCV genome is integrated into the host genome should be determined by using HCV probes other than the present probes.

The sequences of HCV clones from JHH-4 and JHH-6 clearly revealed that there was no contamination with HCV RNA positive serum. Moreover, there is no cross-contamination between JHH-4 and JHH-6, because the sequences of HCV clones differed from each other. The possibility of cross-contamination between HCV RNA positive serum and JHH-1 remained, since the sequences of HCV clones from them were similar. However, the sequence of the 5′UTR is known to be highly conserved, particularly in genotype 1b, which accounts for ~70% of all HCV in Japan [Okamoto et al., 1992c] and is well conserved [Okamoto et al., 1990b, 1992b]. Therefore, the 5′UTR sequences of five HCV clones reported previously, such as HCV-BK [Takamizawa et al., 1991], HCV-J [Kato et al., 1990], HC-J4/83 [Okamoto et al., 1992a], HC-J4/91 [Okamoto et al., 1992a], and HCV-T [Chen et al., 1992], all of which belong to genotype 1b were examined. Four of these clones had the same 5′UTR sequence, except for HCV-J, which had only one different nucleotide in the sequence. Thus most HCV clones that belong to genotype 1b have exactly the same 5′UTR sequence.

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                               -250      -240      -230      -220      -210      -200      -190      -180      -170      -160
                                 |         |         |         |         |         |         |         |         |         |
HCV-1     (genotype 1a):CCATGGCGTTAGTATGAGTGTCGTGCAGCCTCCAGGACCCCCCCTCCCGGGAGAGCCATAGTGGTCTGCGGAACCGGTGAGTACACCGGAATTGCCAGGACGACC
HCV-BK    (genotype 1b):.........................................................................................................
HC-J6     (genotype 2a):........................A...........C...........................................................G...A...T
HC-J8     (genotype 2b):........................A...........C........................................................A..G.A.A...T
HCV RNA positive serum :.........................................................................................................
JHH-1                  :.........................................................................................................
JHH-4                  :........................A...........C........................................................T..G.A.A...T
JHH-6                  :........................A...........C...........................................................G.A.A...T
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Fig. 2. Comparison of nucleotide sequences in the 5′UTR of HCV RNA detected in the three HCC cell lines (JHH-1, JHH-4, and JHH-6). The representative clones of four genotypes (HCV-1, HCV-BK, HC-J6, and HC-J8) are also shown. The sequences are compared with HCV-1 on the top line. Dots indicate sequence identity with HCV-1. The classification of genotypes by Simmonds et al. [1994] has been adopted.

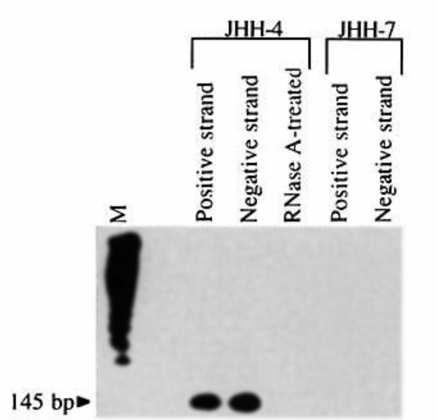

Fig. 3. Detection of intracellular negative-strand HCV RNA by the method of strand-specific RT-PCR. To synthesize cDNA, external antisense primer (#36) or external sense primer (#32) was used for detection of positive or negative-strand HCV RNA, respectively. After reverse transcription, the reaction mixture was heated at 94°C for 3 min and quickly chilled, and then incubated with 100 μg/ml of RNase A at 37°C for 90 min to degrade residual RNA template. Each cDNA was amplified by nested PCR. The second PCR products of each cell line were size-fractionated by electrophoresis in 3% agarose gel and transferred to a nylon membrane. Then they were hybridized with the ^{32}P-labelled probe. As a negative control, the same RNA sample treated with RNase A before reverse transcription was used. RNA extracted from JHH-7 cell line was also used for another negative control. Lane M represents 100 base DNA ladder (Pharmacia). Arrowhead indicates the position of 145 bp DNA fragments, which is the expected size of amplified intracellular HCV RNA.

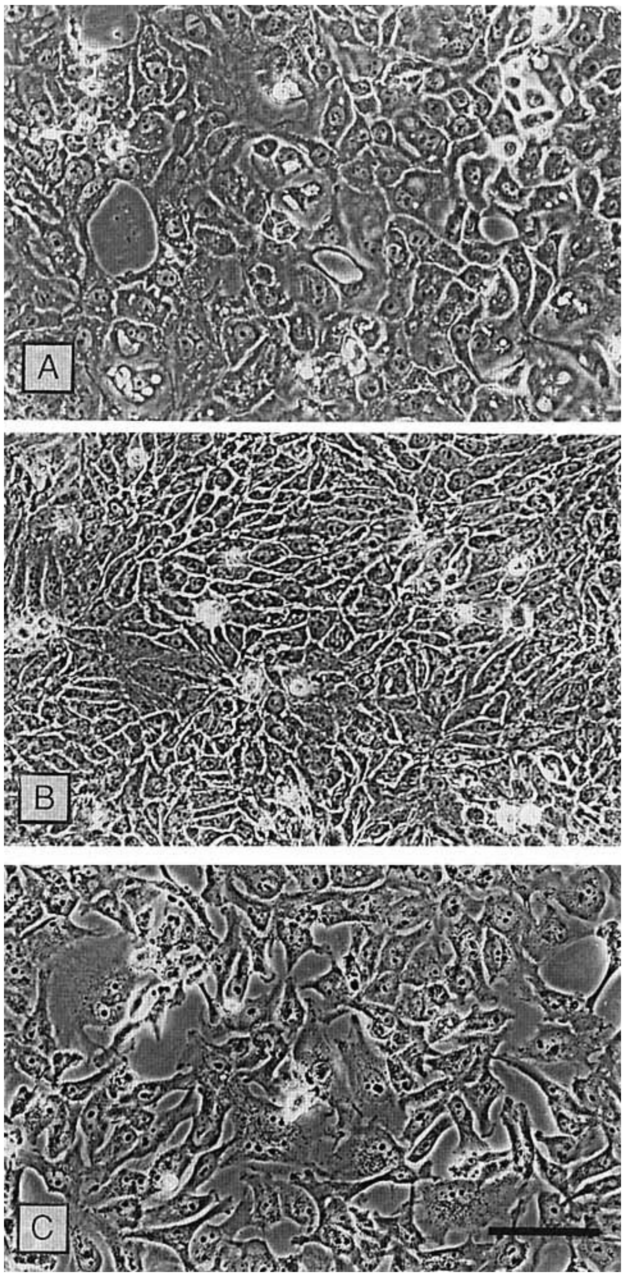

Fig. 4. Phase-contrast photomicrographs of JHH-1 (**A**), JHH-4 (**B**), and JHH-6 (**C**) cells. They are polygonal in shape and resemble hepatocytes. Bar 100 μm.

It was also possible to detect negative-strand HCV RNA in the JHH-4 cell line. Since the negative-strand HCV RNA is a replicative intermediate [Fong et al., 1991; Gunji et al., 1994; Sakamoto et al., 1994; Takehara et al., 1992], HCV in JHH-4 cell line may be replicating, implying that the entire virus genome is present in the cells. However, negative-strand HCV RNA was not detected in JHH-1 or JHH-6. These findings may be due to lower amount of intracellular HCV in these two cell lines than in JHH-4.

Although it was possible to detect repeatedly the 5′UTR of HCV in these HCC cell lines, it was not always possible. Among the tested samples from JHH-1, JHH-4, and JHH-6, 25–48% were HCV-positive. Recently, in situ hybridization experiments demonstrated that the distribution of HCV in the liver was very heterogeneous [Haruna et al., 1993; Lau et al., 1994; Negro et al., 1992]. In other words, there were noninfected tissue areas in the liver, and there were also noninfected hepatocytes in the infected tissue areas. Additionally, virus-infected hepatocytes were found at a much lower frequency in the liver of patients with HCV than in the liver of patients with HBV [Hiramatsu et al., 1992]. Thus a very small number of cells in these HCC cell lines were actually infected with HCV. Shimizu et al. [1992, 1993, 1994] and Shimizu and Yoshikura [1994] demonstrated that the human T-cell lines MOLT-4 Ma and HPB-Ma supported the replication of HCV for >2 months after inoculation with HCV RNA positive serum, but viral RNA was not always detected but was detectable intermittently during the culture. In addition, positive and negative-strand HCV RNA were not always detected at the same time. Nissen et al. [1994] also reported the same phenomenon. These findings are consistent with the present results. The reason why HCV cannot always be detected may be due to an extremely few number of HCV in the cells at the time of harvesting or due to technical failures related to RT-PCR. A standard method by which we can constantly detect HCV in cultured cells must be developed as soon as possible.

In conclusion, among the three HCC cell lines reported here, JHH-4 seems to be the most suitable for HCV studies. There is the possibility that JHH-4 cells may be used as an in vitro system for the propagation of HCV.

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